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Producer-Cell Modification of Human Immunodeficiency Virus Type 1: Nef Is a Virion Protein

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Type 1 human immunodeficiency viruses encoding mutated *nef* reading frames are 10- to 30-fold less infectious than are isogenic viruses in which the *nef* gene is intact. This defect in infectivity causes *nef*-negative viruses to grow at an attenuated rate in vitro. To investigate the mechanism of Nef-mediated enhancement of viral growth rate and infectivity, a complementation analysis of *nef* mutant viruses was performed. To provide Nef in *trans* upon viral infection, a CEM derivative cell line (designated CLN) that expresses Nef under the control of the viral long terminal repeat was constructed. When *nef*-negative virus was grown in CLN cells, its growth rate was restored to wild-type levels. However, the output of *nef*-negative virus during the first 72 h after infection of CLN cells was not restored, suggesting that provision of Nef within the newly infected cell does not enhance the productivity of a *nef*-negative provirus. The genetically *nef*-negative virions produced by the CLN cells, however, were restored to wild-type levels of infectivity as measured in a syncytium formation assay in which CD4-expressing HeLa cells were targets. These *trans*-complemented, genetically *nef*-negative virions yielded wild-type levels of viral output following a single cycle of replication in primary CD4 T cells as well as in parental CEM cells. To define the determinants for producer cell modification of virions by Nef, the role of myristoylation was investigated. Virus that encodes a myristoylation-negative *nef* was as impaired in infectivity as was virus encoding a deleted *nef* gene. Because myristoylation is required for both membrane association of Nef and optimal viral infectivity, the possibility that Nef protein is included in the virion was investigated. Wild-type virions were purified by filtration and exclusion chromatography. A Western blot (immunoblot) of the eluate fractions revealed a correlation between peak Nef signal and peak levels of p24 antigen. Although virion-associated Nef was detected in part as the 27-kDa full-length protein, the majority of immunoreactive protein was detected as a 20-kDa isoform. *nef*-negative virus lacked both 27- and 20-kDa immunoreactive species. Production of wild-type virions in the presence of a specific inhibitor of the human immunodeficiency virus type 1 protease resulted in virions which contained only 27-kDa full-length Nef protein. These data indicate that Nef is a virion protein which is processed by the viral protease into a 20-kDa isoform within the virion particle.

The *nef* gene of primate immunodeficiency viruses is an important determinant of virulence in both experimental infection of adult monkeys and human disease (10, 20). Infection with *nef*-negative virus is associated with low levels of viral replication, preservation of normal levels of CD4-positive T cells, and the absence of disease. Although clearly a positive factor for viral replication in vivo, the development of in vitro models of *nef* function has been complicated by experimental inconsistency (9). *nef* was originally named as an acronym for "negative factor," but the majority of recent data support a positive role for *nef* during viral replication in vitro (8, 11, 21, 24, 33). However, the actual function of the *nef* gene product remains controversial. Currently, three putative functions can conceivably account for the positive effect of Nef: (i) down-regulation of CD4 (the viral receptor) from the cell surface (1, 4, 16, 18), (ii) modulation of T-cell activation events (3, 12, 27, 32, 33), and (iii) enhancement of viral infectivity (8, 24, 25).

Several laboratories have confirmed that *nef* has a positive impact on viral infectivity (2, 8, 24, 30). By using either terminal dilution titration of infectivity to lymphoid cells or an infectious-center assay in which CD4-expressing HeLa cells are targets, *nef*-positive virus was found to be approximately 10-fold more infectious than *nef*-negative virus (8, 24). The relatively poor infectivity of *nef*-negative virus appears to be caused by impaired viral DNA synthesis following entry of virions into newly infected cells (2, 7, 30). These findings indicate that the effect of *nef* on infectivity is manifest prior to viral gene expression in the newly infected cell; Nef apparently modifies the virion in the producer cell.

The virion modification hypothesis has been supported by the following observation: the infectivity impairment of genetically *nef*-negative virions can be rescued by expression of Nef in *trans* during virion production (2, 25). These recent studies on *trans*-complemented *nef*-negative virus have been limited to the analysis of infectivity with CD4-expressing HeLa cells as targets. In the present study, the phenotypic analysis of *trans*-complemented Δ*nef* virus is extended to include viral output during single-cycle infections (with primary cultures of CD4-positive T cells as well as a T-cell line) and to growth rate assays with a T-cell line that expresses Nef only following infection. The data obtained support the conclusion that virion modification by Nef is sufficient to explain the impact of Nef not only on viral infectivity but also on viral output and growth rate in vitro.

The modification of virions that is induced by Nef is un-

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known. *nef*-negative virions are indistinguishable from wild-type virions in their amounts of packaged RNA and in their contents and processing of known viral structural proteins (8, 25). In the present study, we demonstrate that Nef protein is a virion component. Furthermore, we present evidence that the majority of virion-associated Nef is present as a 20-kDa isoform, formed by proteolytic modification of full-length Nef by the virus-encoded protease (14, 15). We hypothesize that the particle modification induced by *nef* is the inclusion of Nef protein in the virion and that virion-associated Nef functions in the recipient cell to enhance infectivity.

MATERIALS AND METHODS

Plasmid construction. For construction of pLTR/nef; to place the *nef* gene under the direct transcriptional control of the 5′ long terminal repeat (LTR), pNL43 was digested with *Bss*HII and *Bam*HI. The digestion product containing the viral LTRs and the *nef* gene was gel purified and treated with Klenow DNA polymerase (Gibco BRL) to blunt the overhangs, before ligation with T4 DNA ligase (Boehringer Mannheim) and transformation of *Escherichia coli*. Plasmid DNAs from the transformants were analyzed by restriction digestion to confirm the presence of the *Bss*HII-*Bam*HI deletion, which creates the pLTR/nef expression vector. For construction of proviral *nef* mutants, pΔnef and pMD were prepared as previously described (8, 33). pΔnef is a mutant of pNL43 containing a deletion between nucleotides 71 and 256 of the *nef* gene and a premature termination codon at nucleotide 272. pMD is a pNL43 mutant containing G-to-C point mutations at positions 8891 and 8894 of pNL43; these changes introduce glycine-to-alanine missense codons in the N-terminal myristoylation signal.

Viruses and cell lines. For generation of the CEM/LTRnef line, pLTR/nef was linearized with *Aat*II and gel purified. Then 0.9 μg of linearized vector and 0.1 μg of pRC/CMV (Invitrogen), which contains the neomycin resistance gene, were mixed with 5 μg of Lipofectin (Gibco BRL). The Lipofectin-DNA mixture was used to transfect 10^6 CEM cells. Transfected cells were aliquoted into 24 wells of a 48-well microtiter plate at a density of 40,000 cells per well. After 2 days of growth in RPMI 1640 medium supplemented as described below, G418 (Gibco BRL) was added to each well to a final concentration of 800 μg/ml. Two weeks later, microcultures containing surviving cells were expanded in the continued presence of 800 μg of G418 per ml. To determine whether the G418-resistant CEM lines expressed the *nef* gene under the control of the human immunodeficiency virus (HIV) LTR, 10^6 cells of each line were either mock infected or infected with 5×10^5 pg of p24 of the genetically *nef*-negative virus, Δnef. Nine days after infection, infected and mock-infected cells were analyzed by Western blot (immunoblot) as described below. Of 11 G418-resistant cell lines, 1 expressed detectable Nef protein only following viral infection. This cell line was named CEM/LTRnef (CLN) and was used in the *trans* complementation experiments.

Parental CEM cells were maintained in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin-streptomycin. The CLN line was maintained in RPMI 1640 medium supplemented as above plus 800 μg of G418 per ml. Cells of the HeLa-T4 line HT4-6C were maintained in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 μg of G418 per ml until used in syncytium formation assays (6). 293 cells were maintained in minimal essential medium with Earle's balanced salts (EMEM) (Quality Biologicals) supplemented with 2 mM glutamine, penicillin-streptomycin and 10% fetal bovine serum.

Stocks of NL43, Δnef, and MD viruses were prepared by transfection of CEM cells as described previously (8). Proviral plasmid DNA (1 μg) of each virus was combined with 5 μg of Lipofectin. This mixture was incubated at 37°C with 10^6 CEM cells in 1 ml of Optimem medium (Gibco BRL) for 5 h. Transfected cells were then supplemented with 3 ml of supplemented RPMI medium. Cultures were split 1:3 every 3 days. After 10 days, when few cells appeared uninfected and the cytopathic effect was extensive, the cultures were centrifuged at $800 \times g$ to pellet the cells and the virus-containing supernatants were aliquoted and frozen at −80°C.

For production of virus stocks by transient transfection of 293 cells, 10^6 cells were plated in each 35-mm well of a six-well plate 24 h prior to transfection. Transfection was carried out by the calcium phosphate method with the CellPhect transfection kit (Pharmacia) as specified by the manufacturer and 3 μg of either pNL4-3, pMD, or pΔNef. At 12 h after transfection, the cells were washed twice with 2 ml of EMEM and once with 2 ml of 150 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)–15% glycerol (pH 7.5) before the addition of fresh EMEM (2 ml per well). At 72 h later, virus-containing supernatants were pooled and gross cellular debris was removed by centrifugation at $800 \times g$ for 10 min. For NL4-3 virus prepared in the presence of the HIV-1 protease inhibitor SC52151 (5), 293 cells were preincubated for 30 min in 5 μM SC52151 in EMEM prior to transfection. The drug remained present during transfection of the cells with 3 μg of pNL4-3 and was added back (to 5 μM) after the washing and medium replacement. The concentration of p24 antigen in all viral stocks was measured by a quantitative enzyme-linked immunosorbent assay (ELISA; Abbott Laboratories).

Infection of CEM and CLN cell lines. Growth curves were initiated by infection as follows. For each infection, 10^6 CEM or CLN cells were pelleted at $800 \times g$. The cell pellet was resuspended in 1 ml of supplemented RPMI 1640 medium containing 5×10^5 pg of p24 from the cell-free virus. The cell-virus mixtures were incubated at 37°C for 2 h, and the cells were washed three times with culture medium. The infected cells were suspended in 4 ml of medium for continued incubation. Every 3 to 4 days, samples of the total culture (cells plus medium) were harvested for p24 ELISA analysis and the cultures were split 1:3.

Infection of primary cultures of CD4-positive lymphocytes. CD4-positive T lymphocytes were purified from the peripheral blood of healthy, HIV-seronegative donors by a negative selection technique involving panning with a mixture of monoclonal antibodies directed to CD8, CD19, and CD16 as described previously (33). The primary CD4 cells were activated by culture for 3 days in RPMI 1640 medium with 5% human AB serum containing 3 μg of phytohemagglutinin (Sigma Chemical Co.) per ml plus 5 U of recombinant interleukin-2 (DuPont, NEN Research Products) per ml. Following activation, the cells were washed and counted; aliquots were infected for 4 h at 37°C with 50,000 pg of p24 per 10^6 cells. Excess virus was removed by three washes with phosphate-buffered saline (PBS), and viable-cell recovery was determined by trypan blue staining. The infected cells were suspended in medium supplemented with 5 U of recombinant interleukin-2 per ml and distributed into 96-well, round-bottom microtiter plates (Linbro Plastics) at 10^5 cells per well in triplicate. To prevent multiple cycles of reinfection during culture, a murine monoclonal antibody with HIV_{LAI}-specific neutralizing activity was added to the microculture wells at a 1:200 dilution (26). Immediately after plating and at 2 and 4 days after infection, HIV replication was monitored by harvesting 100-μl supernatant samples from each well, combining the aliquots from replicate wells, and measuring the concentration of p24 antigen by ELISA.

Infectivity assays. The infectivities of the virus stocks were determined with a CD4-expressing HeLa cell line (HT4-6C) in a syncytium formation assay as described previously (6). Serial fivefold dilutions of each virus stock in Dulbecco's modified Eagle's medium supplemented with 4% fetal bovine serum, 2 mM glutamine, 0.5 μg of Polybrene per ml, and 8 μg of DEAE-dextran per ml were placed over a monolayer of HT4-6C cells in microtiter wells. Three days later, the wells were fixed in methanol and stained with crystal violet, and the syncytia were counted. Wells containing between 70 and 250 syncytia were used to calculate infectivity, which was expressed as picograms of p24 antigen per syncytium-forming unit (SFU).

Purification of virions. For the analysis of the presence or absence of Nef protein, HIV-1 virions were purified as follows. Supernatants from cultures of CEM cells initially transfected with either pNL4-3 or pΔnef and displaying extensive cytopathic effect were obtained by centrifugation at $800 \times g$. Virus-containing supernatant (2 ml, containing 4×10^6 pg of p24) was filtered through a 0.45-μm-pore-size membrane. The filtrate was concentrated by centrifugation at $23{,}500 \times g$ for 1 h at 4°C. The virion pellets were resuspended in 100 μl of supplemented RPMI 1640 medium. The concentrated virion solutions were loaded onto 2.5-ml gel filtration columns containing Sephacryl S-1000 (Pharmacia) (23). Tris-buffered saline (TBS; 2 ml) was added to the column, and the eluate was collected in 100-μl fractions. For NL4-3 virions produced in the presence of the protease inhibitor SC52151, columns were equilibrated with TBS containing 5 μM SC52151. Individual fractions were analyzed by ELISA for their p24 content and subsequently pelleted at $23{,}500 \times g$ for 1 h at 4°C and then resuspended in 10 μl of Western loading buffer (63 mM Tris-HCl [pH 6.8], 12.5% [vol/vol] glycerol, 2% [wt/vol] sodium dodecyl sulfate [SDS], 5% [vol/vol] β-mercaptoethanol, and 0.003% [wt/vol] bromephenol blue).

Western blots. Immunodetection of Nef protein was performed as follows. Samples were resolved in a 4% stacking, 15% resolving polyacrylamide minigel with the Laemmli discontinuous buffer system and electroblotted onto 0.2-μm-pore-size nitrocellulose membranes. The membranes were incubated in Super-Block blocking buffer (Pierce) for 45 min at room temperature. They were then incubated overnight at 16°C in blocking buffer plus a 1:2,000 dilution of a polyclonal sheep antiserum raised to recombinant NL4-3 Nef produced by *E. coli*. The membrane was washed twice for 10 min and once for 20 min in PBS plus 0.1% [vol/vol] Tween-20. Following washing, the membrane was incubated for 1 h in blocking buffer plus 1:25,000 donkey anti-sheep antibody conjugated to peroxidase (Binding Site). Immunoreactive product was detected by enhanced chemiluminescence with the ECL Western blotting detection system (Amersham International). Exposure times varied from 30 s to 1 min.

RESULTS

Design of a *trans*-complementation system for HIV-1 *nef* mutants. Cell-free wild-type HIV-1 is more infectious than isogenic mutants in which the *nef* gene is inactivated (8, 24). The relatively poor infectivity of *nef*-negative mutants is caused by impaired viral DNA synthesis following entry of the virion into newly infected cells (2, 7, 30). These findings have led us to investigate whether provision of Nef in *trans* to genetically *nef*-negative virions during their production can (i) augment

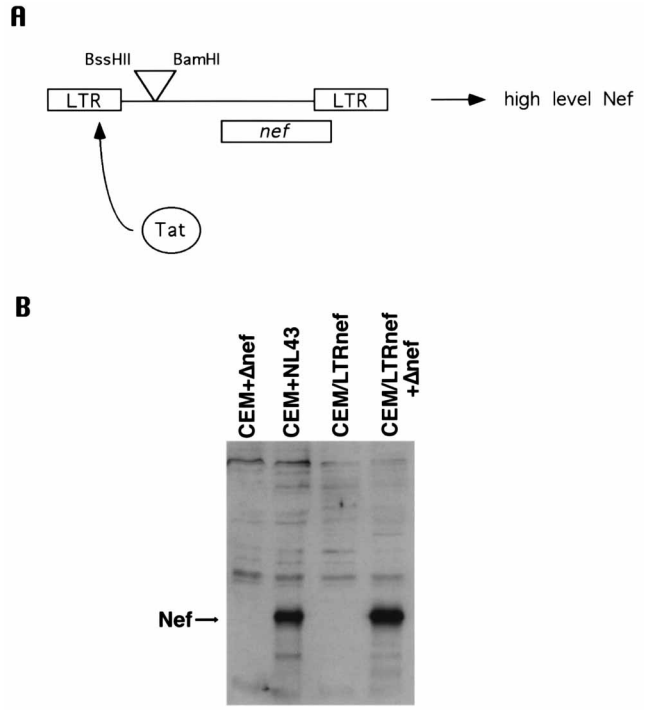

FIG. 1. (A) Schematic of the LTR-*nef* expression construct which was stably introduced into CEM cells to create the cell line CEM/LTRnef (CLN). Tat protein, provided in *trans* following viral infection, will activate the expression of Nef. (B) Western blot of parental CEM cells and CLN cells infected with either Δnef or NL4-3 virus. Cells (10^6) were infected with virus (5×10^5 pg of p24). Approximately 10^5 cells of each infected culture were harvested on day 10 and analyzed for expression of Nef protein by Western blotting as described in Materials and Methods.

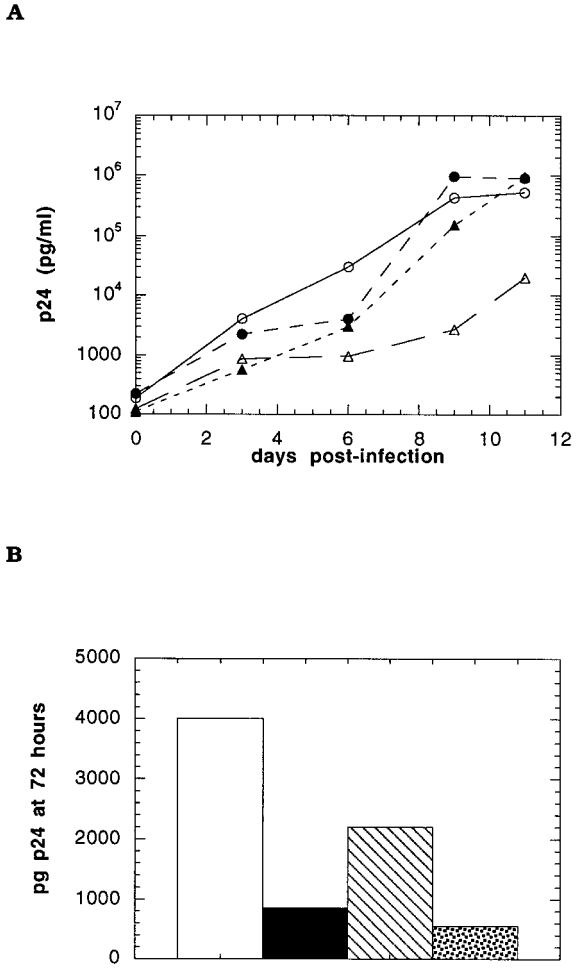

FIG. 2. (A) Growth curves of Δnef and NL4-3 viruses grown in either parental CEM cells or CLN cells. In each case, 5×10^5 pg of p24 from each virus was used to infect 10^6 cells. Cultures were split 1:3, and samples were harvested for p24 assay every 2 to 3 days. Symbols: ○, NL4-3 in the parental CEM line; ●, NL4-3 in the CLN line; △, Δnef in the parental CEM line; ▲, Δnef in the CLN line. (B) p24 output at 72 h after infection of parental CEM cells and CLN cells with NL4-3 and Δnef virus. These data are from the day 3 time point of panel A but are shown here on an arithmetic scale. Symbols: □, NL4-3 in CEM cells; ■, Δnef in CEM cells; ▨, NL4-3 in CLN cells; ▩, Δnef in CLN cells. These data are representative of three experiments.

their infectivity to recipient cells, (ii) restore their viral output to wild-type levels during a subsequent single cycle of replication, and (iii) restore their growth rate to wild-type levels.

To address these questions, the vector pLTRnef, which contains the NL4-3 *nef* gene under the transcriptional control of the HIV-1 LTR, was constructed. This vector was stably introduced into CEM cells, yielding the cell line CLN. Cells of this line express detectable amounts of Nef only following viral infection (Fig. 1). This system allows for expression of Nef within infected cells in coordination with other early HIV-1 genes. It also allows for the propagation of viruses in cultures of Nef-expressing CD4-positive T cells; the problem of CD4 downregulation, which would prevent viral spread within cultures of cells that express Nef constitutively, is avoided (4).

Complementation of viral growth rate but not initial viral output by Nef expression in recipient cells. The CLN line enabled the investigation of how *nef* affects the growth rate as well as the infectivity of HIV-1. First, we compared the growth rates of a virus containing a deletion in the *nef* gene (Δnef) and wild-type virus (NL4-3) in the CLN line and in parental CEM cells (Fig. 2A). Growth curves of these two viruses in parental CEM cells showed a disparity in relative growth rate; wild-type virus eventually produced 100-fold more p24 antigen than did Δnef. When the same experiment was performed with the CLN line, in which Nef protein is provided in *trans* in the infected cells, the growth rate of Δnef virus was restored to wild-type levels.

Two possibilities might account for the equalized growth rates of wild-type and Δnef virus in cultures of CLN cells. This effect might be caused by restoration of the infectivity of Δnef virions to wild-type levels by provision of Nef during virion production. Alternatively, Nef expression in the recipient cell might increase the production of p24 antigen from the newly established provirus. To distinguish between these possibilities, the output of p24 antigen by wild-type and Δnef viruses following a single cycle of replication in CEM and CLN cells was compared (Fig. 2B). To approximate a single cycle of viral replication, the production of p24 antigen during the initial 72 h following infection was measured. Δnef virus produced approximately fourfold less p24 antigen than did wild-type virus, regardless of which line was used as the source of recipient cells. These data suggested that provision of Nef in *trans* when linked to Tat-mediated transactivation in the recipient cell does not augment the production of p24 antigen from a newly established provirus. These data are consistent, however, with the hypothesis that the equalized growth rates of *nef*-positive and *nef*-negative viruses seen when Nef is provided in *trans* (Fig. 2A) are caused by augmentation of the infectivity of Δnef virions.

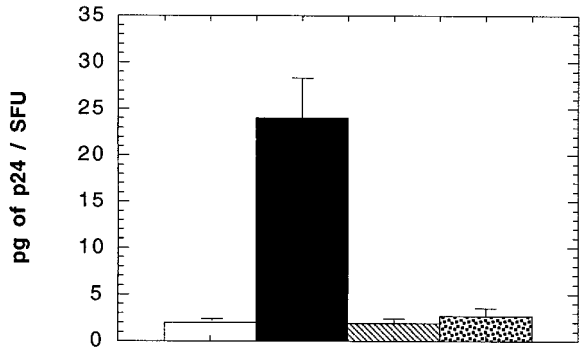

FIG. 3. Effect of the provision of Nef protein within producer cells on virion infectivity. NL4-3 and Δnef viruses were produced by transfection of either CEM or CLN cells with equal amounts (1 μg) of plasmid proviral DNA. Cultures were maintained for 8 days, whereupon virions were harvested and used to infect HeLa-T4 cell monolayers in a syncytium formation assay as described in Materials and Methods. SFU: syncytium-forming-unit. Symbols: □, NL4-3 virions produced by parental CEM cells; ■, Δnef produced by parental CEM cells; ▨, NL4-3 virions produced by CLN cells; ▨, Δnef virions produced by CLN cells. The error bars indicate the standard deviation of two infectivity values obtained from independently produced viral stocks; each infectivity value was the average of the number of syncytia counted in duplicate wells infected with each viral stock.

Complementation of infectivity by Nef expression in producer cells. To confirm the hypothesis that Nef modifies the virion to enhance infectivity, we measured the infectivities of *nef*-positive and *nef*-negative viruses produced either by parental CEM cells or by CLN cells. The infectivity of these viruses was measured by a quantitative syncytium induction assay with CD4-expressing HeLa indicator cells (HeLa-T4 cells) (6). The amount of p24 antigen required for an SFU for these viruses is shown in Fig. 3. When viruses were produced by parental CEM cells, *nef*-negative (Δnef) virus was 10- to 20-fold less infectious than the wild type, requiring 10- to 20-fold more p24 antigen for an SFU. When these viruses were produced by CLN cells, the infectivities of the Δnef and wild-type viruses were identical to each other and equal to that of wild-type virus produced by parental CEM cells (Fig. 3). These data confirm that provision of Nef in *trans* during virion production restores the infectivity of genetically *nef*-negative virions to wild-type levels (2, 25). Interestingly, the provision of potentially supraphysiological amounts of Nef protein to the wild type during propagation in the CLN line did not further augment its infectivity.

Virion modification by Nef enhances viral output during the subsequent cycle of replication. Next, we investigated the growth rate dynamics of *trans*-complemented Δnef viruses in parental CEM cells (Fig. 4). These experiments were designed to answer two questions. First, is the output of p24 antigen by *trans*-complemented Δnef virions following a single cycle of replication in parental CEM cells restored to wild-type levels? Second, is this effect transient; that is, do *trans*-complemented Δnef virions revert phenotypically and display a reduced growth rate over time in accordance with their *nef*-negative genotype? To answer the first question, the productions of p24 antigen 72 h after infection of parental CEM cells with either *trans*-complemented Δnef virus, noncomplemented Δnef virus, or wild-type virus were compared (Fig. 4A). The output of p24 antigen by cells infected with *trans*-complemented Δnef virus was similar to that of cells infected with the wild-type viruses, and all viruses that were produced in the presence of Nef generated more p24 antigen than did Δnef (Fig. 4A). To answer the second question, these infected cultures were maintained to generate growth curves for these viruses in parental

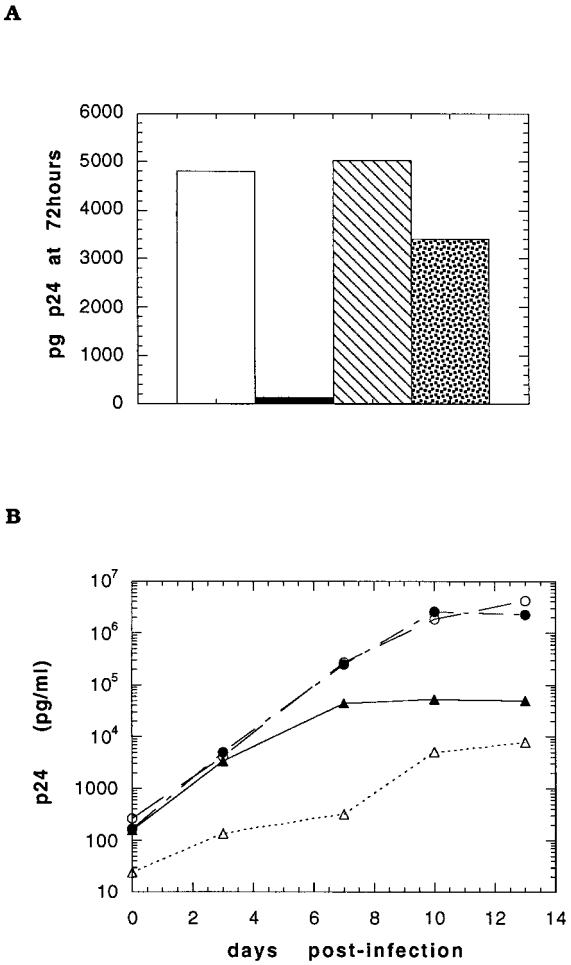

FIG. 4. (A) Effect of provision of Nef protein within producer cells on viral output during the subsequent cycle of replication. NL4-3 or Δnef viruses (5×10^5 pg of p24) produced by either CEM cells or CLN cells were used to infect 10^6 CEM cells. At 72 h following exposure of the cells to the virus, samples of each culture were harvested and analyzed for p24 content. Symbols: □, NL4-3 produced by CEM cells; ■, Δnef produced by CEM cells; ▨, NL4-3 produced by CLN cells; ▨, Δnef produced by CLN cells. (B) Growth curves of NL4-3 and Δnef viruses originally produced by either CEM cells or CLN cells and then propagated in parental CEM cells. These curves represent the continuation of the infection described in panel A. Symbols: ○, NL4-3 produced by CEM cells; △, Δnef produced by CEM cells; ●, NL4-3 produced by CLN cells; ▲, Δnef produced by CLN cells. These data are representative of two experiments.

CEM cells (Fig. 4B). As these viruses propagated, the growth rate of *trans*-complemented Δnef became attenuated relative to that of the wild-type viruses. These data indicated that the infectivity and output characteristics of *trans*-complemented Δnef virus were restored for only a single viral replicative cycle in recipient cells; thereafter, its growth rate reverted to match its *nef*-negative genotype. Titer determination of the viruses produced during the infections shown in Fig. 4 with the CD4-HeLa assay revealed that *trans*-complemented Δnef virus also reverted phenotypically to a characteristically low infectivity after passage in parental CEM cells (data not shown). These data document phenotypic reversion and indicate that the modification of Δnef virus induced during production by CLN cells is not due to genetic restoration of the *nef* gene. This conclusion was confirmed by reverse transcription-PCR of the *nef* gene in *trans*-complemented Δnef virions; the size of the

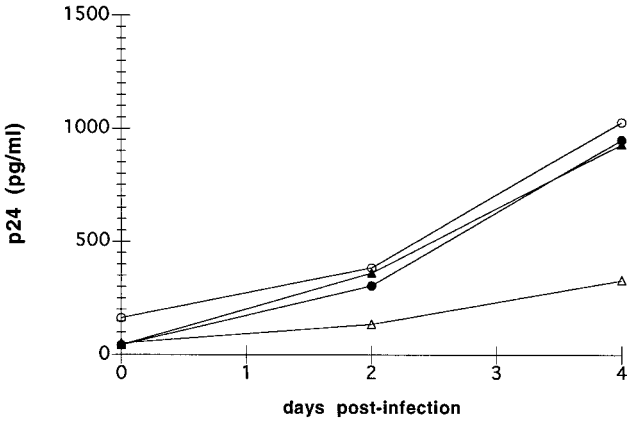

FIG. 5. Single-cycle viral output by NL4-3 and Δnef virions produced in the presence of Nef protein and used to infect primary T lymphocytes. CD4-positive primary T lymphocytes (10^6) were prestimulated with IL-2 and PHA before infection with 5×10^4 pg of p24 from each virus as described in Materials and Methods. After exposure of the cells to virus, neutralizing antibody was added to limit the infection to a single cycle of replication. Symbols: ○, NL4-3 produced by CEM cells; △, Δnef produced by CEM cells; ●, NL4-3 produced by CLN cells; ▲, Δnef produced by CLN cells.

PCR products indicated the continued presence of the deletion in *nef* (data not shown).

Virion modification by Nef enhances viral output from primary cultures of CD4-positive lymphocytes. *nef*-negative virus grows at an attenuated rate in primary cultures of CD4-positive T cells (24, 33). To determine whether *trans* complementation by provision of Nef in the producer cell is sufficient to restore the production of p24 antigen from newly infected primary T cells, prestimulated CD4-positive lymphocytes obtained from peripheral blood were infected with the viruses described in Fig. 4. To ensure a single cycle of replication, neutralizing antibody was added after exposure of the cells to the viruses (8). As shown in Fig. 5, Δnef virus produced fourfold less p24 antigen than did wild-type virus. However, the output of p24 by *trans*-complemented Δnef virus was restored to wild-type levels. These data suggested that the modification which renders *trans*-complemented Δnef virions more infectious to recipient cells can manifest itself within the context of primary T cells.

Myristoylation is required for Nef-mediated enhancement of infectivity. To begin to identify the requirements for Nef-mediated modification of the virion, the phenotype of a myristoylation-negative *nef* mutant was studied. Nef is normally myristoylated at its N terminus; this modification is necessary for the association of Nef with cellular membranes (19). Myristoylation-deficient (MD) viruses were produced by parental CEM cells and by CLN cells. MD virus was characterized by an infectivity deficit comparable to that of Δnef virus (Table 1). Interestingly, the infectivity of *trans*-complemented MD virus was restored to wild-type levels; in this case, both the wild-type *nef* gene product and the MD *nef* gene product presumably coexisted during virion production. These data suggested that myristoylation deficiency is not a dominant negative mutation. More importantly, these data imply that Nef protein must associate with membranes during virion production to enhance infectivity.

Detection of Nef protein in the virion. The dependence of Nef-mediated enhancement of infectivity on myristoylation suggests that Nef protein may be incorporated into virion particles. To examine this possibility, a purification scheme was designed to separate virion particles from Nef-containing cellular debris which may contaminate viral stocks derived from cytopathic infections. Virion-containing supernatants were obtained by centrifugation of acutely infected cultures of CEM cells at $800 \times g$. These crude supernatants were filtered through 0.45-μm-pore-size membranes to further remove cellular debris and then concentrated by centrifugation at 23,500 $\times g$. The concentrated virions were then subjected to exclusion chromatography on a Sephacryl S-1000 matrix. This matrix has a spherical exclusion size of approximately 400 nm. Although the size of an HIV-1 virion is approximately 150 nm, the peak of viral p24 antigen eluted within or near the void volume of the column (data not shown). When the fractionated virion preparations were analyzed both by an ELISA assay for p24 antigen and by Western blotting for Nef protein, protein species immunoreactive with Nef antibody were detected in the fractions containing the peak concentrations of the capsid protein p24 (Fig. 6A). These data suggested that Nef is a virion protein. Interestingly, full-length, 27-kDa Nef made up a relatively minor fraction of the virion peptides reactive with Nef antibody. The majority of the Nef-specific signal was found in a doublet of approximately 20 kDa and a smear migrating in the 10- to 14-kDa range. These Nef-related peptides are reminiscent of the products that result from in vitro cleavage of recombinant Nef protein by recombinant HIV-1 protease (14, 15).

TABLE 1. Infectivities of wild-type and *nef* mutant viruses assessed by syncytium formation on HeLa-T4 cells

Virus[a]	Source	Mean infectivity[b] (pg/SFU) ± 1 SD
NL4-3	CEM	8 ± 4
NL4-3	CLN	12 ± 2
Δnef	CEM	60 ± 15
Δnef	CLN	16 ± 6
MD	CEM	279 ± 42
MD	CLN	22 ± 7

[a] Viral stocks were prepared by transfection of the indicated cell line and harvest of the culture supernatants after 8 days.
[b] Infectivity was measured as described in Materials and Methods and in the legend to Fig. 3. Mean infectivity was calculated from four independent determinations.

To define further the efficacy of this purification procedure, we performed an add-back experiment in which cellular debris containing Nef (but no virions) was mixed with Δnef virions prior to purification and analysis as described above. Cells of the line CEM4B, which express Nef in the absence of virion structural proteins from a *rev*-defective provirus (22), were disrupted by hypotonic shock before the addition of Δnef virions. After removal of cells by centrifugation at $800 \times g$, Nef, presumably associated with cellular debris from the CEM4B cells, was readily detectable by Western blotting (Fig. 6B). After filtration through 0.45-μm membranes, however, almost all detectable Nef was removed (Fig. 6B). After column chromatography, no Nef was detected in the fractions containing the p24 antigen derived from the Δnef virions (Fig. 6C). These data suggested that Nef detected in our purified preparations of wild-type virions is unlikely to derive from contamination with cellular debris.

Nef protein is cleaved by the HIV-1 protease within virion particles. To determine whether the approximately 20-kDa protein in virion preparations recognized by Nef-specific antiserum was the result of processing of full-length Nef by the virally encoded protease, we prepared virions by transient

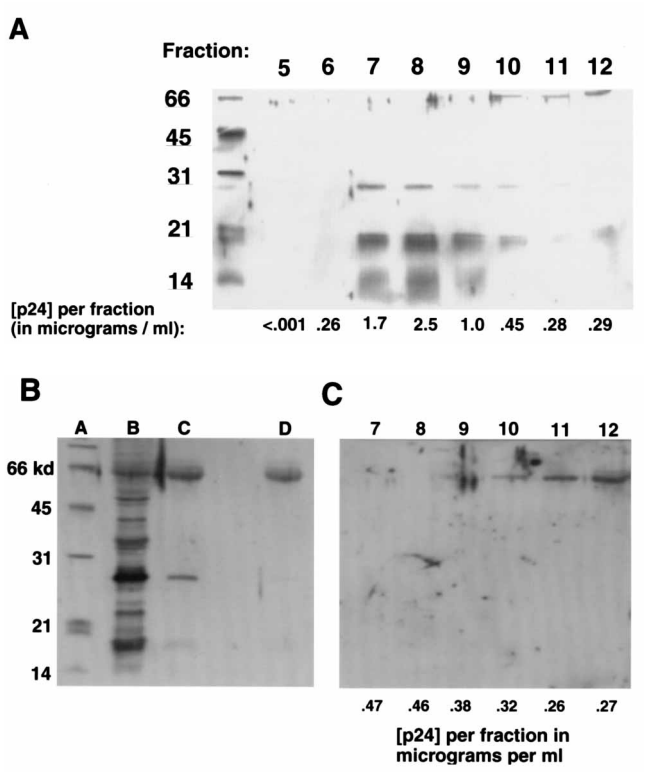

FIG. 6. Virion association of Nef protein. (A) Western blot of NL4-3 virions purified by gel filtration chromatography followed by protein detection with a sheep polyclonal antibody to recombinant Nef protein. NL4-3 virus (4×10^6 pg of p24) harvested from acutely infected CEM cells was filtered through a 0.45-μm membrane, pelleted at 23,500 × *g*, and then resuspended in 100 μl of medium before being loaded onto a 2.5 ml, S-1000 Sephacryl gel filtration column as described in Materials and Methods. Fraction numbers refer to the 100-μl aliquots of eluate collected sequentially after addition of TBS to the column. Each fraction was analyzed for p24 content by ELISA; these values are shown below each lane. Virions in each fraction were pelleted at 23,500 × *g* and loaded onto a 4% stacking, 15% resolving SDS-polyacrylamide gel. The far left lane contains molecular weight markers (indicated on the left in thousands). The gel was electroblotted and probed for Nef as described in Materials and Methods. (B). Western blot for Nef protein showing the effect of filtration on signals derived from cell-associated Nef protein. Lanes: A, molecular mass markers; B, CEM4B cells (5×10^4), which express Nef in the absence of virions from a *rev*-defective provirus, after lysis in hypotonic medium (0.1×); C, CEM4B cells (1.5×10^6) after lysis in 1 ml of hypotonic medium, removal of cellular debris by centrifugation at 800 × *g*, and concentration of the supernatant by centrifugation at 23,500 × *g* (one-tenth of the pellet was loaded onto the gel); D, lysate of CEM4B cells prepared identically to that described for lane C, except that the lysate was first filtered through a 0.45-μm membrane. (C). Western blot for Nef protein using a preparation of Δnef virions that had been spiked with the Nef-containing hypotonic lysate of CEM4B cells prior to purification. Δnef virions (3×10^6 pg of p24) in 900 μl were added to 100 μl of hypotonic lysate from 1.5×10^6 CEM4B cells. The virion-Nef–containing cell-lysate mixture was then filtered through a 0.45-μm membrane, pelleted at 23,500 × *g*, resuspended in 100 μl of medium, and loaded onto a Sephacryl S-1000 gel filtration column. Fractions (100 μl) were collected, analyzed for p24 content, and centrifuged at 23,500 × *g* prior to SDS-polyacrylamide gel electrophoresis. The numbers at the top of each lane represent the fraction number from the gel filtration column. The p24 concentrations of the fractions are shown below each lane.

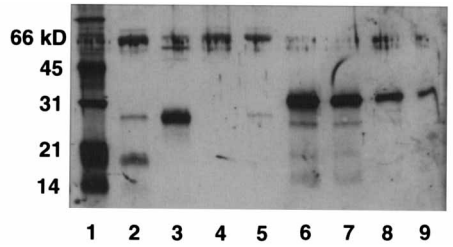

FIG. 7. HIV-1 protease cleaves virion-associated Nef protein. Shown is a Western blot of purified virion preparations generated by transient transfection. 293 cells (10^6) were transfected with 3 μg of pNL4-3 (in the presence or absence of the protease inhibitor SC52151), pΔNef, or pMD. After 72 h, virus-containing supernatants were purified by gel exclusion chromatography as described in the legend to Fig. 6; fractions containing peak p24 were pooled and quantitated. Lanes: 1, molecular mass standard marker; 2, NL4-3 virions, no drug; 3, NL4-3 virions prepared in the presence of 5 μM SC52151; 4, ΔNef virions; 5, MD Nef mutant virions. Lanes 2, 4, and 5 were equalized by p24 content (30,000 pg per lane), while lanes 2 and 3 were equalized by RNA content ($\approx 6 \times 10^8$ copies as measured by the Roche Amplicor, HIV-Monitor Assay). Lanes 6 to 9 contain recombinant Nef protein (5, 2.5, 1, and 0.5 ng, respectively); the recombinant Nef is of greater apparent molecular mass than the native Nef protein owing to the presence of an amino-terminal polyhistidine tag.

transfection of 293 cells with pNL4-3 in the presence and absence of a highly specific inhibitor of the HIV-1 protease, SC52151 (5). These virions, in addition to Δnef and MD virions (also prepared from cultures of 293 cells), were purified by filtration and gel exclusion chromatography as described above. Peak fractions of each virion species were pooled and quantitated by measurement of their p24 content. Equal amounts of each virion stock were subjected to SDS-gel electrophoresis and Western blotting for Nef protein (Fig. 7, lanes 2 to 5). For wild-type virus produced in the presence of protease inhibitor, the RNA content was used to standardize the amount of virus loaded, because the p24 assay appeared to underestimate the total amount of virus produced in the absence of active protease (data not shown). NL4-3 virions produced by 293 cells in the absence of SC52151 (lane 2) yielded a pattern of immunoreactive bands very similar to that of CEM-derived, purified virions (Fig. 6A), consisting of both 27- and 20-kD bands. NL4-3 virions produced in the presence of 5 μM SC52151 (Fig. 7, lane 3) lacked the protein which corresponds to the 20-kDa band. However, these virions contained an increased amount of 27-kDa Nef protein per particle. This indicated that the 20-kDa immunoreactive protein within NL4-3 virion preparations is a product of cleavage by the HIV protease. Virions which do not encode Nef protein (Δnef [lane 4]) or which encode a Nef protein that is not actively targeted to the membrane (MD [lane 5]) did not contain any detectable amount of the 20-kDa cleavage product. These results indicated that the 20-kDa product is processed Nef protein, derived by cleavage of full-length Nef by the viral protease. The trace amount of 27-kDa, full-length Nef detected in MD virions was presumably due to small amounts of cellular, nonmembrane-associated Nef in the preparation.

An estimate of the amount of Nef protein present per viral particle can be made by comparing the band intensities of the wild-type virions (Fig. 7, lane 2) with the intensities of the bands of known amounts of recombinant Nef protein (lanes 6 to 9). Such an estimation should be made cautiously, because the antibody used for detection was raised to a full-length, recombinant Nef protein and not to the cleavage product of Nef. Nevertheless, there are probably less than 70 molecules of either full-length or cleaved Nef protein per virion.

DISCUSSION

nef-negative HIV-1 grows at a reduced rate relative to wild-type virus in T-lymphoid cell lines such as CEM and in primary cultures of CD4-positive T lymphocytes (8, 33). The attenuated growth in T-cell lines is caused by impaired viral infectivity (8). The infectivity of *nef*-negative virus is about 10-fold lower than that of wild-type virus, and this impairment causes a 3- to

8-fold decrement in viral output following a single cycle of replication (7, 8).

In the present study, we show that each of these virologic phenotypes of *nef*-negative virions can be effectively *trans* complemented by provision of Nef during virion production. Using a CEM-derivative cell line named CLN that encodes *nef* under the control of the HIV LTR to provide Nef protein in *trans*, we show restoration not only of impaired cell-free viral infectivity but also of the impaired viral output following single-cycle replication and the impaired growth rate of *nef*-negative HIV-1. Complemented viral output following single-cycle replication is shown not only with the CEM line but also with primary cultures of CD4-positive T lymphocytes as recipient cells. We confirm that myristoylation of Nef is required for the virion modification that enhances infectivity (17). Finally, we demonstrate that Nef protein is incorporated into virions and, once incorporated, is processed by the viral protease to yield a 20-kDa fragment.

Two studies have shown previously that provision of Nef in *trans* can augment the infectivity of genetically *nef*-negative virions (2, 25). In both these studies, transient-transfection methods were used to produce virions from *nef*-negative proviruses in the presence or absence of cotransfected Nef expression vectors. Both studies used CD4-expressing HeLa cells as indicator cells to measure viral infectivity. However, a potential caveat to these observations is the reported discrepancies between these infectivity assays and growth rate assays (24, 30); although *nef*-negative HIV-1 uniformly shows impaired infectivity in HeLa-T4-based assays, an impaired growth rate in T-cell lines has not been a consistent finding among different laboratories (8, 21, 24, 30). Therefore, it was important to determine whether the virion modification induced by Nef in the producer cell was sufficient to explain the viral output and growth-rate-enhancing effects of *nef*. The data reported herein indicate that Nef-mediated modification of the virion is sufficient not only for infectivity enhancement as measured by HeLa-T4-based assays but also for the augmentation of viral output and the restoration of a wild-type growth rate to genetically *nef*-defective HIV-1. In addition to allowing these output and growth rate assays, the CLN cell line described herein provided a reproducible complementation system for HIV-1 *nef* mutants. This complementation system will be important for future mutagenesis studies of *nef*; for many mutants, it will be necessary to prove by *trans* complementation that the observed phenotypes are not due to mutations in the overlapping LTR.

The virion modification by Nef which increases viral output during a subsequent single cycle of replication was evident not only in CEM cells but also when primary cultures of CD4-positive lymphocytes were used as recipient cells. Interestingly, an attenuated growth rate during replication in primary T cells is another phenotype of *nef*-negative HIV-1 which has been observed consistently in different laboratories (11, 24, 33). The question has remained, however, whether enhancement of infectivity and enhancement of growth rate in primary cells are causally related phenomena. For primary T cells that have been stimulated to undergo cell division prior to infection, modification of the virion by Nef was sufficient to restore wild-type levels of viral output to *nef*-negative virus. This finding essentially excludes any augmentation of viral production caused by Nef expression from the newly formed provirus in prestimulated recipient cells. Instead, virion modification by Nef in the producer cell appears sufficient to explain all the virologic phenotypes examined.

What, then, is the virion modification that is induced by Nef? Previous studies and the data herein indicate that N-terminal myristoylation (and presumably membrane association) is critical to the virion modification induced by Nef (17). The simplest hypothesis that accounts for this relationship is that Nef is a virion protein. Data presented herein indicate that Nef is indeed a virion protein. We hypothesize that virion-associated Nef acts in the recipient cell to increase the likelihood of successful infection. As shown previously, the impact of *nef* in the recipient cell is manifest as enhanced viral DNA synthesis following viral entry (2, 7, 30). Accordingly, Nef protein contained in virions may facilitate the synthesis of viral DNA in recipient cells either directly or in concert with cellular factors.

Proteolytic processing of Nef by the HIV-1 protease in vitro yields a 19-kDa, well-folded C-terminal fragment which contains virtually all of the conserved structural motifs in Nef with the exception of the N-terminal myristoylation signal (13, 14); these motifs include an acidic domain, an SH3-binding domain, and residues essential for the binding of Nef to cellular serine kinases (28, 29, 31). We speculate that this 19-kDa fragment is the same as or very similar to the 20-kDa Nef isoform detected within virion particles in this study. This 20-kDa isoform may be responsible for the action of Nef in recipient cells, either by interacting directly with processes such as virion uncoating or reverse transcription or by transducing a signal that creates a more favorable environment for viral DNA synthesis in the newly infected cell. Preparation and subsequent analysis of viral mutants that do not exhibit cleavage of Nef in virions will aid in discerning whether inclusion of Nef protein and its subsequent cleavage within the virion particle are required for infectivity enhancement.

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